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Immunocytochemical and Ultrastructural Study of Localization of the γ-Aminobutyraldehyde Dehydrogenase in the Bovine Brain

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γ-Aminobutyraldehyde dehydrogenase (γ-ABALDH), is an enzyme which catalyses the dehydrogenation of γ-aminobutyraldehyde (ABAL) to γ-aminobutyric acid (GABA), an inhibitory neurotransmitter in the mammalian central nervous system. To investigate the ultrastructural location of γ-ABADL in bovine brain, immunocytochemical staining for electron microscopical observation was done using anti-γ -ABALDH antibodies. Immunoreactive particles with high electron density were observed in the perikarya of neurons. In unmyelinated and thin myelinated nerve fibers, highly electron dense materials were observed not only around the microtubules and mitochondrial membrane, but also in the mitochondrial matrix. Cells containing γ-ABALDH, found in many regions of bovine brain tissues, were mostly small and medium-sized neurons, generally known as interneurons. Ultrastructurally, the γ-ABALDH was determined to be distributed mainly in the cytoplasm and around the membranes of the endoplasmic reticulum and the mitochondria.

γ-Aminobutyric acid (GABA), a 4 carbon amino acid, was first discovered in brain tissue in 1950 (Awapara *et al.*, 1950; Roberts and Frankel, 1950), and is well known to be the major inhibitory neurotransmitter in mammalian CNS (Grillo, 1985). GABA is produced mainly through enzymatic decarboxylation of glutamic acid by glutamic acid decarboxylase (GAD) (Wu, 1976; Roberts, 1979) and also synthesized by the degradation of putrescine (Caron *et al.*, 1988). GABA is also an inhibitory neurotransmitter in the mammalian CNS (Seiler and Al-Therib, 1974; Larsson *et al.*, 1984). Recently, antibodies to GABA itself have been produced (Storm-Mathisen *et al.*, 1983; Hodgson *et al.*, 1985). Antibodies to GABA can be used in revealing clearly the GABAnergic axon terminals and GABA-containing neurons. GABA-containing neurons can be observed in some brain areas such as the neocortex, thalamus, hippocampus and cerebellar cortex, and localization of GABA and GAD in the brain is well known (Ottersen and Strom-Mathisen, 1984; Somogyi *et al.*, 1985).

Aldehyde dehydrogenases catalyse the irreversible oxidation of a broad range of aldehydes to the corresponding acids in an NAD^+-dependent reaction (MacKerell and Pietruszko, 1987). In human liver, aldehyde dehydrogenase isozyme 1 (ALDH1) and isozyme 2 (ALDH2) has been found to be located in the cytoplasm and the mitochondria, respectively (Maeda *et al.*, 1988). In addition, it has been reported that in horse, rat, and bovine liver, ALDH1 is also located in the cytoplasm and ALDH2 in the mitochondria of hepatocytes (Tottmar *et al.*, 1973; Koivula, 1975; Eckfeldt and Yonetani, 1976; Greenfield and Pietruszko, 1977). A third isozyme of human liver aldehyde dehydrogenase (EC 1.2.1.3) was found to be γ-aminobutyraldehyde dehydrogenase (Larsson *et al.*, 1984; Sternberger, 1986).

Putrescine (1,4-diaminobutane), a well-knowm polyamine, degrades to GABA via the intermediate γ-aminobutyraldehyde (γ-ABAL) (Tabor and Tabor, 1984; Pegg, 1986). Putrescine thus provides the substrate γ-ABAL for the synthesis of GABA by γ-aminobutyraldehyde dehydrogenase (γ-ABALDH). Studies on aldehyde dehydrogenases with low Km values for γ-ABALDH have been carried out extensively for the past several years (Kurys *et al.*, 1989; Lee and Cho, 1992, 1993). The degradation pathway of putrescine has been detected in rat brain and liver (Jänne *et al.*, 1978; Pegg, 1986), mouse brain, and some other tissues (Grillo, 1985). The conversion of putrescine into GABA seems to be very important in such tissues described above. However the enzymatic specificity of this enzyme has been known to act toward many sub-

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The abbreviations used are: γ-ABAL, γ-aminobutyraldehyde; γ-ABALDH, γ-aminobutyraldehyde dehydrogenase; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; PAP, peroxidase-antiperoxidase; PBS, phosphate buffered saline.

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strates including γ-ABAL, suggesting low specificity. Recently, we first isolated and characterized γ-ABALDH with a much higher specificity toward γ-ABAL (Lee and Cho, 1992).

Clearly, γ-ABAL could be a critical regulator of neuron activity. However, little information is available concerning the localization in the bovine brain except some properties reported by us (Lee and Cho, 1992, 1995). Using the immunocytochemical staining method for electron microscopy, we have shown that this enzyme, unexpectedly is widely distributed. The other properties concerning this enzyme such as expression will be published in a forthcoming paper (Lee and Cho, 1995).

Materials and Methods

γ-Aminobutyraldehyde dehydrogenase from bovine brain was purified by using CM-trisacryl, DEAE-Sephacel, 5′-AMP-Sepharose affinity chromatography, and fast protein liquid chromatography (FPLC) on a Mono-Q column (Pharmacia) (Lee and Cho, 1992). During purification, enzyme activity was assayed in a solution containing 0.1 M K^+-phosphate buffer (pH 8.0), 5 mM NAD^+, 0.5 mM DTT, and 2 mM γ-ABAL. All assays were performed spectrophotometrically at 340 nm (Jakoby and Fredericks, 1959). Protein concentration was determined as described by Lowry *et al.* (1951) with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

The anti-γ-ABALDH antiserum was prepared from a rabbit immunized subcutaneously with γ-ABALDH. Serum samples were tested by immunodiffusion, and the precipitates of the antigen-antibody reaction were identified. Antisera used for immunocytochemical staining was purified using a protein A column (Harlow and Lane, 1988).

Isolated bovine brains were immersed immediately in a fixation solution (4% paraformaldehyde solution, 0.1 M phosphate buffered, pH 7.4), and preserved in a fixation solution (3% glutaraldehyde, 3% paraformaldehyde, 0.1% picric acid solution, 0.1 M phosphate buffered, pH 7.4) for 20 h at 4 ℃. Tissues were trimmed according to the atlas of Lauder (1982), sectioned into 50 μm thickness by a vibratome, and immunostained by the method of Sternberger (1986). Before the immunostaining, tissues were washed with 0.1 M phosphate buffered saline (PBS) in order to remove the fixation solution, and incubated in a 1% sodium borohydride-0.1 M PBS solution for 30 min. After washing with 0.1 M PBS, tissues were pretreated for 10 min in a 3% hydrogen peroxide solution, for 1 h in 10% normal goat serum (NGS, Gibco Lab., New York) at room temperature, and then washed by 0.1 M PBS (pH 7.4, containing 0.1% Triton X-100). They were incubated in the PBS solution containing anti-bovine rabbit γ-ABALDH antibody for 72 h at 4 ℃, and washed. They were then incubated in the solution containing goat anti-rabbit IgG (secondary antibody) for 2 h, and in the PAP (peroxidase-antiperoxidase) solution for 1 h at room temperature. They were stained by 0.05% diaminobenzidine (DAB, in 0.05 M tris-HCl, pH 7.6, containing 0.01% hydrogen peroxide) and washed with 0.1 M PBS. All incubation steps were performed in a humidified chamber. Electron microscopical preparations were made according to general tissue preparation procedures.

For electron microscopic observation, the immunostained tissues were washed in a phosphate buffer and placed in 1% osmium tetroxide (0.1 M phosphate buffer) for 30-60 min. The tissues were then rinsed in maleate buffer (pH 6.0), stained with uranyl acetate in maleate buffer, washed, dehydrated, and embedded with EPON mixture (Coulter, 1967). Selected areas were trimmed and mounted on a EPON chuck with a drop of EPON. The thin sections were cut by ultramicrotome and mounted on the copper grids and observed without lead citrate staining with a Philips electron microscope.

Results and Discussion

The anti-γ-ABALDH antiserum was prepared from a rabbit immunized subcutaneously with γ-ABALDH. Serum samples were tested by immunodiffusion and western blotting, and it was determined that the antibodies formed in the rabbit were specific to the purified enzyme.

Through immunohistochemical staining, anti-γ-ABALDH immunoreactive neurons were found in many regions of bovine brain tissues such as the precentral gyrus (motor cortex), postcentral gyrus (sensory cortex), occipital cortex (visual cortex), olfactory area, hippocampus, thalamus, caudate nucleus, lentiform nucleus, and cerebellum. Anti-γ-ABALDH immunoreactive cells were mostly small-sized and medium-sized neurons, generally known as interneurons. These results coincided with the previous reports that most inhibitory neurons are interneurons. Immunoreactive particles were found in the cytoplasm of the nerve cells. The results of the electron microscopic observations were not confined only within the nucleus but located outside of the nucleus. Based on the immunostaining results, γ-ABALDH also exists in GABAnergic neurons, leading to another synthetic pathway. γ-ABALDH has been reported as a cytosolic enzyme, but there have been no reports regarding its intracellular localization. Therfore immunocytochemical staining for γ-ABALDH was performed in order to determine its ultrastructural location using the anti-γ-ABALDH antibody.

In the electron microscopic observation, γ-ABALDH distribution was mainly observed in the cytoplasm. Immunoreactive particles with high electron density were observed in the perikarya of some neurons (Fig. 1A), however there were some other neurons in which immunoreactive particles were not observed (Fig. 1B). The positive structures were found mainly around the

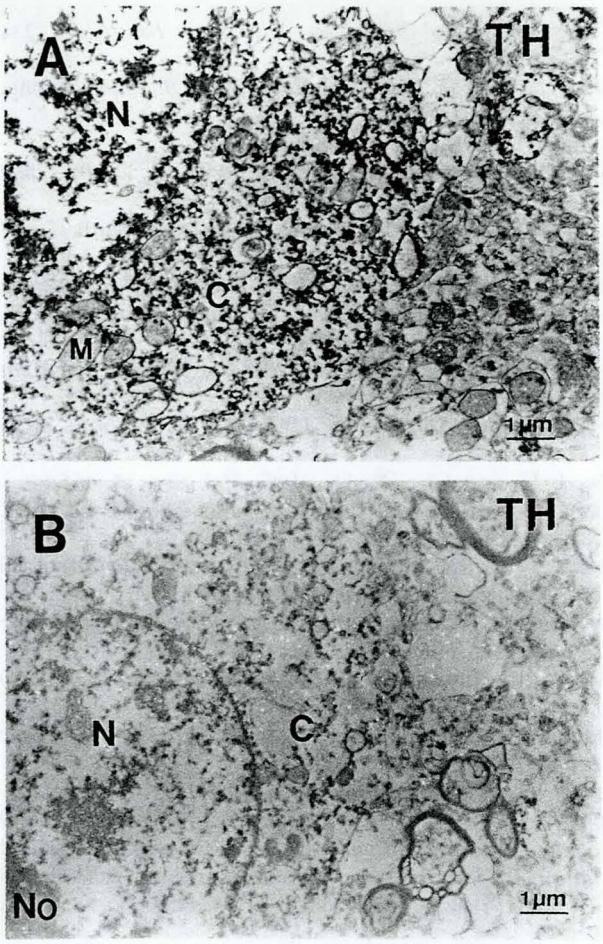

Figure. 1. Electron micrographs of the immunoreactive structure in the thalamus of bovine brain. A, Immunoreactive particles with high electron density were observed in the perikarya of some neurons. B, There were some other neurons in which immunoreactive particles were not observed. TH, thalamus; N, nucleus; No, nucleolus; M, mitochondria; C, cytoplasm.

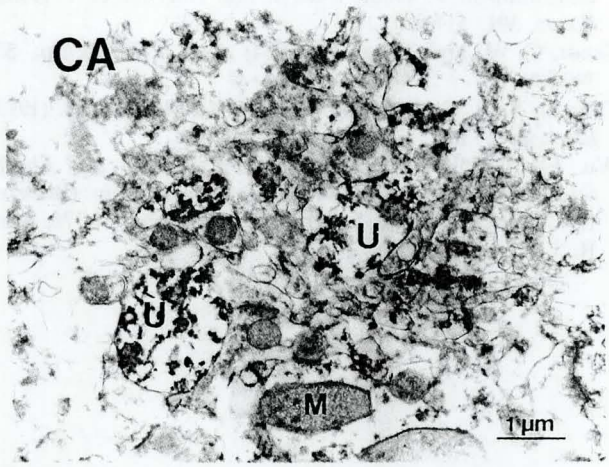

Figure 2. Electron micrograph of the immunoreactive structure in the caudate nucleus (CA) of bovine brain. Many immunoreactive unmyelinated fibers were observed. U, unmyelinated axon fiber; M, mitochondria.

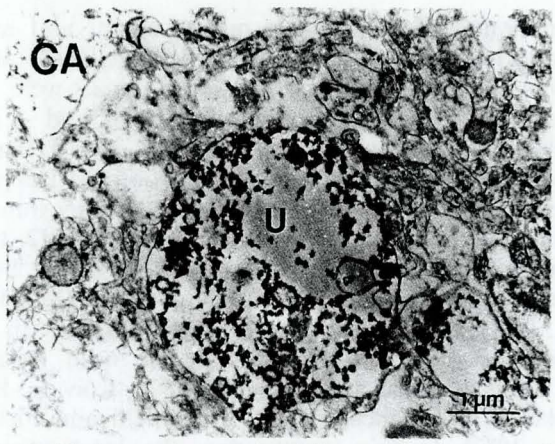

Figure 3. Electron micrograph of the caudate nucleus (CA) in bovine brain. γ-Aminobutyraldehyde dehydrogenase-immunoreactive structures (→) are observed around microtubules in the unmyelinated axon. U, unmyelinated axon fiber.

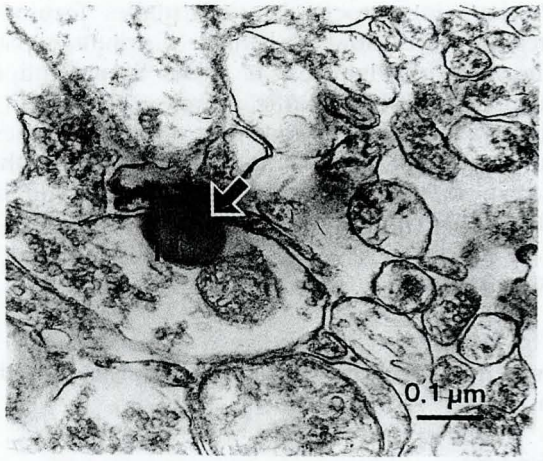

Figure 4. Electron micrograph of the immunoreactive structure in the thalamus of bovine brain. Immunoreactive structures are distributed around the mitochondrial membrane. M, mitochondria.

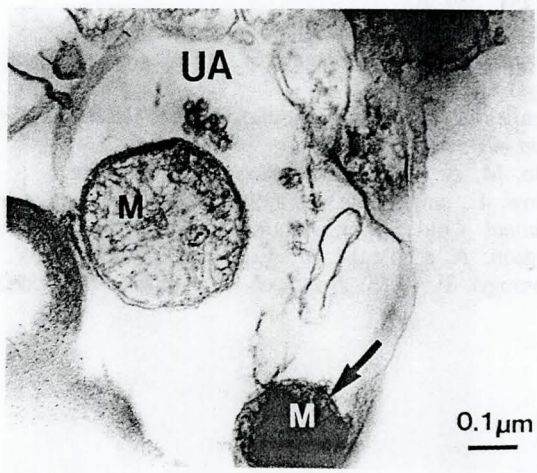

Figure 5. Electron micrographs of thalamus in bovine brain. Immunoreactivity was strong within the mitochondrial matrix of the unmyelinated axon. M, mitochondria; UA, unmyelinate axon.

membranes of the endoplasmic reticulum and mitochondria. In unmyelinated and thin myelinated nerve fibers, high electron dense positive materials were also observed around the microtubules (Figs. 2 and 3), and mitochondrial membrane (Fig. 4) and matrix (Fig. 5). Localization of the aldehyde dehydrogenase isozymes have been investigated in the human liver by immunostaining. Human liver ALDH1 and ALDH2 have already been determined to be localized in the hepatocyte cytoplasm and nucleus, respectively (Maeda *et al.*, 1988). The localization of these enzymes in the horse, rat, and bovine liver has also been reported as being the same (Tottmar *et al.*, 1973; Koivula, 1975; Eckfeldt and Yonetani, 1976; Greenfield and Pietruszko, 1977). In this investigation, the localization of γ-ABALDH, reported as being the third isozyme of ALDH in the human liver, was determined. γ-ABALDH of bovine brain was located in both cytoplasm and mitochondrial matrix of neurons.

Previous immunocytochemical studies focused on putrescine have showed a similar distribution pattern to the above results (Lee *et al.*, 1991). Strong antiputrescine-immunoreactive particles were observed in the perinuclear cytoplasm and near the membranes of many organelles. These results correlate well with the fact that putrescine is a substrate for the γ-aminobutyraldehyde dehydrogenase.

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